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Angiogenesis is a basic process in the development of normal tissues and organs. Excessive and insufficient angiogenesis is directly involved in maintaining and development of some severe diseases. Maybe the best known example of excessive angiogenesis is the tumor-associated formation of new blood vessels that on one hand provide nutrients for cancer cells, and on the other gives rise to a network that allow the spreading of malignant cells, as Judah Folkman has shown in experimental models five decades ago [1]. The hypothesis that newly formed blood vessels are essential for tumor progression and metastasis has been hardly accepted in a period when almost only tumor cells were investigated and considered as target for therapy. The discovery and characterization of the first and most powerful angiogenic factor [2], as well as of the first antiangiogenic drug [3] dramatically changed our perspective on angiogenesis and antiangiogenesis. Since these moments, a lot of researches focused on angiogenesis as a potential prognostic factor in human tumors and as a target for therapy. Destroying and/or normalizing the vascular network it was hoped to reduce tumor dimensions and to inhibit local and regional spreading [4].

Methods to investigate and evaluate angiogenesis are very different, and even the same method applied by different groups gave controversial results. One example is microvessel density, performed virtually by almost all researchers in the field. Since 1990’s when Weidner [5] described the best known and most applied method to evaluate microvessel density, its prognostic value has been demonstrated in almost all human malignant tumors. Even so, the values published by different groups are significantly different, including the works based on microscopic image analysis. The same conclusion applies to other clinico-pathologic and experimental methods used to investigate tumor-associated angiogenesis. This is why standard procedures and guidelines to apply and to interpret technical methods is not necessary, but mandatory, and it has been recently published in the journal Angiogenesis [6]. This is a major achievement as validation of different antiangiogenic drugs became already a necessity.

What do we know nowadays? We know that the vascular network is crucial for tumor progression, tumor-associated blood vessels are different in structure and function from vessels found in normal tissues, and endothelial cells show a high rate of proliferation. This process is governed by growth factors, namely vascular endothelial growth factors (VEGF), platelet-derived growth factors (PDGF), fibroblast growth factors (FGF), and their cognate receptors. To the promotion and development of angiogenesis also contribute other growth factors and substances that are less investigated or their effect(s) is not enough demonstrated. It is supposed that growth factors act together at the same time or in cascade to induce formation of new blood vessels. What we do not know? We do not know why in some advanced stage malignant tumors there is a low expression of growth factors and their receptor, although they show a rich vascular supply. It is not known why in some tumors blood vessel maturate quickly, and intermediate and immature vessels represent only a minority. Most probably the answer could be given by other growth factor or angiogenic substances that wait to be discovered. We need a better characterization of endothelial cells from tumor-associated blood vessels, including tip cells [6]. Otherwise, a targeted antivascular therapy without damaging normal vessels would be not possible.

A major revolution in antiangiogenic therapy was the approval in 2004 of bevacizumab by Food and Drug Administration, for patients with metastatic colorectal carcinoma [7]. The approval was based on excellent and promising results obtained in both in vitro and in vivo experiments. From that moment, there were produced a lot of humanized monoclonal antibodies, which targeted growth factors, their receptors, additional molecules, a.o. We know that in some patients bevacizumab significantly improves disease-free survival and overall survival in combination with chemotherapy. On the other hand, clinical results are far to be spectacular as initially believed. Why the failure of antiangiogenic therapy? Maybe some tumors do not express the targeted growth factor…, maybe tyrosine kinase inhibitors did not “match” with the chemotherapeutic regimen…, and maybe patients were not stratified according to the angiogenic molecular profile…

What we do not know and we should? Most probably to evaluate the effects of antivascular in addition to the antiangiogenic therapy. This should be performed in patients with angiogenic tumors, characterized...
predominantly by immature and intermediate vessels with endothelium characterized by a high rate of proliferation, associated to VEGF/PDGF/FGF expression at protein level by tumor cells. Although Jimmy, a child in 1991, when he was treated by Folkman with the first antiangiogenic drug for a massive hemangioma [3], was a therapeutic success. Nowadays, it seems that we are not sure which the real place of antiangiogenic therapy is in the anti-cancer therapeutic strategy. It is not clear if a single antiangiogenic drug against a single or multiple target(s) could be the therapeutic option. How to combine antiangiogenic drugs with chemotherapy to obtain better results? These are questions that still wait an answer. We really hope the next coming researches, including those from this journal, will bring new insights of basic mechanisms of angiogenesis and antiangiogenesis in order to understand how to use them in clinical practice.

REFERENCES

Endometriosis represents the presence of endometrial tissue, composed of glands and/or stroma, outside of the uterine cavity, being different from “adenomyosis”, a term used to indicate the profound myometrial disposition of areas with endometrial aspect. It is a benign estrogen-dependent gynecological disease, involving the female genital tract components (uterus, uterine tubes, ovaries, broad, round, and utero-sacral ligaments), in 75% of cases, and extraperitoneal locations, in 25% of cases. From the histopathological point of view, three commonly recognized types are: peritoneal, endometriotic cyst, and deep infiltrative lesions. Although known for a long time, the etiopathogenesis of endometriosis remains incompletely elucidated and its diagnosis is often one of exclusion. Numerous theories have been proposed in order to explain the pathogenic mechanisms, which include: retrograde menstruation, direct mechanical implantation, coelomic metaplasia, proliferation induction, embryonic remnants, vascular and lymphatic dissemination, and stem cells involvement [1].

MATERIAL AND METHODS

Our research comprised of a retrospective study performed on two groups of patients. The first group was represented by 91 cases of endometriosis (ovarian endometriotic cysts and cutaneous abdominal wall endometriosis), diagnosed in the Histopathology Laboratory of the 3rd Clinic of “Elena Doamna” University Clinical Hospital, Iași and the second group by 21 cases of endometrioid or clear cell ovarian carcinomas, diagnosed in the Histopathology Laboratory of the Regional Oncology Institute, Iași during January 2005 - April 2017. The study included the information provided by the patient files as well as the histopathological investigations performed on biological samples - human tissue material obtained during surgical interventions (biopsy, cystectomy, ovariectomy, adnexectomy, hysterectomy with adnexectomy, total radical hysterectomy with pelvic lymphadenectomy) paraffin-embedded and stained by routine histopathological techniques. For all the cases included in the study, patients’ informed consent had been obtained.

RESULTS

A. Group I

The study group involved 91 cases represented by patients aged 20 to 59, with an average of 37.56 years. The distribution by age group is illustrated in Figure 1.

The clinical parameters investigated were: dysmenorrhea, chronic pelvic pain, infertility, dyspareunia, and menometrorrhagia (Figure 2). Dysmenorrhea, the pain associated with menstrual period, was reported...
by 60 of the 92 patients, representing 65.93% of cases. Chronic pelvic pain was reported in 57 cases (62.63%), infertility in 37 cases (40.65%), dyspareunia in 32 cases (35.16%), and menometrorrhagia in 18 cases (19.78%).

Chronic pelvic pain was reported in 57 cases (62.63%), infertility in 37 cases (40.65%), dyspareunia in 32 cases (35.16%), and menometrorrhagia in 18 cases (19.78%).

The performed surgical interventions in Group I were: right, left, or bilateral oophorectomy, bilateral salpingectomy, right, left, or bilateral adnexectomy, cystectomy, tumor excision or biopsy, total or subtotal hysterectomy, and combinations of these.

From the total of 91 cases, 12 (13.18%) were cutaneous abdominal wall endometrioses of the post-caesarean scar (Figure 3), and 79 cases (86.81%) were uni- or bilateral ovarian endometriotic cysts (Figure 4), from which 7 cases (7.69%) were associated with other locations (2 cases with salpingeal and 5 with cervical endometriosis), as shown in Figure 5.

Among the associated histopathological lesions, the most common were (Figure 6): leiomyomas or uterine leiomyomatosis (29.60%), adenomyosis (16.48%), chronic cervicitis (16.48%), endometrial hyperplasia (7.69%), and chronic endometritis (4.39%). In six cases, associated carcinoma was revealed by microscopy: four cases were represented by endometrioid ovarian carcinoma (6.58%) and two cases by endometrioid endometrial carcinomas (2.19%). One case of endometrioid endometrial carcinoma has been synchronous with an endometrioid ovarian carcinoma, with extensive invasion of both organs. The remnant associated pathologies were occasional, being represented by: angiolipoleiomyoma, dermoid cyst, serous ovarian cyst, mesothelioma, oophoritis, polycystic ovary, cervical polyp, endometrial polyp, and salpingitis, each diagnosed in single cases.

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![Figure 1. Distribution by age in Group I](image1)

![Figure 2. Clinical parameters registered in Group I](image2)

![Figure 3. Abdominal wall endometriosis (H&E x10)](image3)
B. Group II

The second group comprised of 21 cases of carcinomas, in which total radical hysterectomy and pelvic lymphadenectomy were performed. Patients were 44 to 74 years of age, with an average of 60.26 years (Figure 7).

Following the histopathological examination (Figure 8), 9 cases of endometrioid carcinoma (Figure 9), 8 cases of clear cell carcinoma (Figure 10) and 4 cases of mixed epithelial tumors (Figure 11 and 12) were diagnosed.

Ovarian endometrioid carcinomas were characterized by an endometrioid-like epithelium, stratified columnar, non-mucinous, with focal villoglandular pattern, with variable intervening tumoral stroma, in an analogous manner to that of endometrial counterpart. Several architectural patterns were observed, such as: papillary, cribriform, (micro)glandular, with ciliated cells, and with sex-cords features. They were graded as well, moderately, or poorly differentiated.

Among the nine cases of ovarian endometrioid carcinomas, one case also showed clear cells areas, one case was associated with areas of atypical endometriosis, and another case associated areas of intraepithelial carcinoma.

Among the eight cases of clear cell carcinoma, two cases also showed areas of serous carcinoma and another case presented areas exhibiting a progression from benign, to borderline, intra-epithelial, up to endometrioid carcinoma.

In any of these cases, where areas showing another phenotype exceeded the 10% threshold, they were considered mixed ovarian epithelial tumors.
Between the cases of mixed ovarian epithelial tumors (four cases), two of them exhibited areas of both endometrioid carcinoma and serous carcinoma, a case of predominantly serous ovarian carcinoma associated clear cell carcinoma areas, and another case showed endometrioid carcinoma areas associated with areas of sex-cord features and also zones of borderline sero-mucinous tumor.

In four of the investigated cases (19.04%), belonging to each tumor category, other benign, borderline, or intraepithelial lesions associated with the invasive component could be identified.

Regarding the tumor grading, 57.14% were poorly differentiated, followed by moderately differentiated carcinomas (23.8%), while 9.5% were well differentiated, and in 9.5% of cases the grading could not be evaluated (Fig. 13).

The distribution of cases according to the FIGO staging and TNM classification (Fig. 14) was performed, showing the following distribution: 52% FIGO stage III and stage FIGO I and II, each of 24%.

DISCUSSIONS

Endometriosis is a benign condition with an incidence that appears to be steadily increasing, probably due to progress in the field of diagnostic techniques and their increasing accessibility.

Both surgical therapy and medical methods fail to achieve etiological treatment, being rather symptomatic. In this context, symptoms recurrence and persistence are
frequent, making this disease a disabling condition. The most severe complication of endometriosis is the possibility of malignancy development. Nevertheless, endometriosis may be ameliorated in its evolution, during pregnancy, and its progression is stopped by menopause onset.

Endometriosis is diagnosed after menarche initiation, when endometrial cells pass via the fallopian tubes into the peritoneal cavity, leading to the development of viable ectopic implants. These would undergo an involution similar to the eutopic endometrium counterpart, at menopause.

Regarding the age of diagnosis, the patients’ age ranged between 20 and 59, in Group I, with an average of 37.56 years. It should be underlined that 49 patients, representing 53.26% of the cases, were aged between 20 and 40, being considered as representing the age of peak fertile period. This high prevalence is largely

![Figure 12. Serous component of a mixed ovarian epithelial tumor associated to ovarian endometriosis (H&E x10)](image)

![Figure 13. Tumor grading in Group II](image)

![Figure 14. Distribution of carcinoma cases according to FIGO staging in Group II](image)
thought to register due to the hormonal influences in the etiopathogenesis of endometriosis and the involvement of the menstrual tubal reflux. Our results also emphasize the importance of an early diagnosis and treatment prior to fertility impairment, as well as the requirement of efficient therapeutic tools that do not interfere with patients’ reproduction capacity. Supplementary, disabling pain caused by endometriosis sometimes leads to a poor quality of life, impossibility to achieve career goals, and reduced work productivity. Moreover, given the many side effects of anti-inflammatory medication, the implementation of an etiological treatment could also avoid symptomatic therapy down-sides.

Patients in Group II were aged 44 to 74 years, with an average of 60.26 years, while the mean age of patients in Group I diagnosed with carcinoma was 54 years.

Even though there is a difference between the average ages between these two groups, there is an even larger difference between these and the mean age of carcinoma-free endometriosis patients (60.26 years and 54 years, respectively vs. 37.56 years). This discrepancy may support the hypothesis that cumulative carcinogenetic effects extended up to several decades are involved in malignancy development on a background of endometriosis.

A recent study on 33 patients involving patients diagnosed with clear cell and endometrioid ovarian carcinomas [2] showed an average of 47.7 years (patients’ age ranged between 31 and 74 years), lower than when compared to our results, possibly due to genetic, racial, geographic, or environmental particularities that predispose to early malignancy development, or by the limited number of patients available to be included in both studies (21 vs. 33).

The most common symptoms of endometriosis registered in our study have been dysmenorrhea (65.93%) and chronic pelvic pain (62.63%), both of them being nonspecific, leading to a delayed diagnosis. Infertility, associated in 40.65% of our cases, highlights the importance of an early treatment, in order to prevent the associated impaired fertility. Our findings are consistent with literature reports, e.g. dysmenorrhea in 62%, chronic pelvic pain in 57%, deep dyspareunia in 55%, cyclic intestinal symptomatology in 48%, infertility in 40% and invalidating dysmenorrhea in 28% of cases [3].

In order to facilitate the clinical diagnosis, the following manifestations are considered predictive of endometriosis: abdominopelvic pain, dysmenorrhea, menorrhagia, infertility, dyspareunia, and postcoital bleeding, along with previous diagnosis of ovarian cyst, pelvic inflammatory disease, and irritable bowel syndrome [4].

The histopathological findings highlight endometriotic cyst as the most common type of lesion (79.12%), followed by cutaneous endometriosis in post-caesarean scar (13.18%), cervical endometriosis (5.49%), and tubal endometriosis (2.19%), the latter two being diagnosed alongside endometriotic cysts, in multifocal endometriosis (7.69%). The location of ectopic implants, multiple sites, and their association with other benign or malignant diseases of the genital tract support one or more of the theories developed so far.

Considering the retrograde mechanical implantation theory (Sampson’s theory), this has been based on the retrograde menstruation mechanism, with reflux of endometrial cells through fallopian tubes to ectopic sites, where the endometrial tissue will attach and then implant. It was proposed for the first time by Sampson, in 1927, and subsequently supported by studies demonstrating retrograde menstruation in women with permeable fallopian tubes [5].

Additionally, the theory of coelomic metaplasia has suggested that peritoneal cells can differentiate into endometrial cells [6], under the influence of unknown factors, endorsed by the fact that endometrial and peritoneal cells have a common precursor, specifically the coelomic cell.

Furthermore, the theory of induction of endometrial proliferation has been a development of the previous theory, suggesting influences of some yet unknown factors contained in the menstrual fluid on peritoneal cells [7], which further induce coelomic cells transformation into endometrial tissue.

Another theory is based on embryonic remnants, supporting the origin of endometriosis in embryonic stem cells and is compatible with the development of lesions in the recto-vaginal septum [8].

In order to support the extrapulmonary development of endometriosis [9], the theory of vascular and lymphatic metastasis (Halban’s theory) suggested the dissemination of endometrial cells through blood vessels and lymphatic vessels.

A more recent theory suggests the involvement of stem cells from either endometrium, either bone marrow, or either endothelial progenitors, which may differentiate into endometriotic tissue at different sites [10].

Although the retrograde menstruation or/and the stem cell theory may support the presence of endometrial tissue in the peritoneal cavity, the development of implants has to be also attributed to several permissive factors which allow their development, such as immunodeficiency, peritoneal attachment capacity, epithelial invasion, vascular network growth, and ectopic tissue survival. Recent molecular studies have been directed towards genetic predisposition, estrogen dependence, progesterone resistance, and inflammation, leading to a current consensus on the association of endometriosis with a chronic inflammatory process induced and/or sustained by the peritoneal microenvironment [11].

Considering, all the possible mechanisms from literature, the predominance of endometriotic cysts in our study (79.12% of cases in group I) demonstrates the ovarian susceptibility for ectopic implantation and supports the involvement of the mechanism of
retrograde menstruation in its development.

Furthermore, abdominal wall endometriosis registered in our study (13.18% of Group I) is possibly the consequence of direct mechanical implantation during cesarean surgery and suggests the existence of immunological factors that allow the survival and development of endometrial implants at this level. This hypothesis underscores the need to improve surgical techniques in order to minimize wound contamination with endometrial cells.

Moreover, less common locations of endometriosis registered in our study, specifically salpingal endometriosis, diagnosed in two cases, supports the tubal menstrual reflux theory, while cervical endometriosis, diagnosed in five cases, suggests the possibility of mechanical implantation, both of which also require the association of immunological disturbances to allow the development of these ectopic implants. In addition, multifocal endometriosis registered in our study (7.69% of Group I cases) supports the association of retrograde menstruation with other mechanisms, based on the development of embryonic remnants or metaplastic processes [12].

Finally, all the mechanisms considered in our study group required the existence of endometrial stem cells, of possible different origins, in order to assure the viability of the implanted tissue.

The analysis of the associated histopathology diagnoses reveals an increased incidence of leiomyoma or uterine leiomyomatosis (29.60% of cases in Group I), probably due to the influence of common etiopathogenical hormonal factors. Considering the similar context of adenomyosis development, in 16.48% of our cases, this finding is further suggesting a local invasive capacity of the endometrium, consistent with literature data [13].

The association with endometrial hyperplasia (7.69% of cases in Group I) is consistent with the same influence of hormonal imbalance in the development of both types of lesions [14].

The association with chronic non-specific cervicitis (16.48%), endometritis (4.39%), salpingitis (1.09%), and oophoritis (1.09%) suggests the influence of immunological factors which are involved in both conditions, as proposed in literature [15].

The association with ovarian endometrioid carcinoma (4.39% of Group I cases), underlies the occurrence of malignant neoplasia on the background of endometriotic lesions [16], with or without an intermediate stage of atypical endometriosis (with progressive accumulation of genetic mutations) [16]. Furthermore, our study supports the probability of an atypical transition phase by our findings (19.04% cases in Group II had atypical endometriosis associated with ovarian carcinoma).

The prevalence of ovarian cancer is higher in women with endometriosis than in the general population [17], considering the common risk factors (early menarche, late menopause, short intervals between menstruations, and nulliparity) and protective factors for both diseases (increased parity, use of oral contraceptives, tubal ligation, and hysterectomy) [18]. Tubal ligation has been strongly associated with a risk reduction for the development of non-serous ovarian neoplasms, particularly endometrioid and clear cell carcinomas, in endometriosis and, in addition, an argument in favor of the retrograde menstruation theory etiopathogeny [19].

In endometriosis-associated ovarian carcinomas (Group II), the nuclear grading and the percentage of the solid areas in endometrioid type were used as criteria for grading. In cases of an undifferentiated pattern of ovarian carcinoma, the following criteria favored an endometrioid carcinoma: metaplastic changes, eosinophilic or secretory cellular phenotype, and fibrous stroma.

Clear cell carcinomas in Group II, automatically graded as G3, have been characterized by the following histopathological features: “clear” appearance of the cell cytoplasm (resulting from glycogen accumulation) or eosinophilic appearance (oxyphilic cells), and hyperchromatic nuclei, bulging into glandular lumen, consistent with a specific “target” pattern. The architectural phenotypes noted were variable, such as: tubulo-cystic/cystic (dilated cystic glands lined by a flattened epithelium), papillary (small, rounded, papillary axes, lined by up to two polygonal or cuboidal epithelial cells layers), solid pattern, and hyalinized, eosinophilic, fibroblastic, or mixoid stroma, consistent with literature data [20].

More than half of the ovarian carcinomas associated to endometriosis have been G3, compared to a minor percentage of G1 (9.5%), corresponding to a less favorable prognosis, and could not be evaluated in cases in which surgery had been performed in post-adjuvant therapy settings (9.5%). Considering that most of the cases have been less differentiated (G2 and G3) (80.22%), we may speculate that ovarian carcinomas developed in a background of endometriosis may also have another type of cancer stem cell origin, when compared to the endometrium counterpart.

The results obtained in Group II, associating various types of ovarian carcinomas, are in line with literature data. In ovarian carcinomas, endometrioid or mixed epithelial tumors associated with an endometrioid component were more common than those with clear cell components in the investigated case (43% vs. 38%). Our findings are consistent with literature data, which estimates endometriosis-associated endometrioid carcinoma as representing 25% of ovarian carcinomas, while clear cell carcinoma accounting only for 5% of these [21]. However, clear cell carcinomas were more common in Group II than described in the literature (38% vs. 25%), but the difference can be attributed to the relatively small number of patients included in our study group.

In order to justify the malignant transformation of endometriosis, several conditions were considered as necessary, namely that the tumor should be adjacent to an endometriotic lesion, with no other primary tumor detected, and the identification of a transition area between the neoplastic epithelium or stroma and the endometriotic component [22, 23]. Nevertheless, given the tendency of malignancies to extend and cover other associated benign or borderline lesions, these may be often absent in surgical specimens. However, in our investigated cases from each category of tumor types (19.04% of cases of Group II), either atypical endometriosis, either borderline lesions,
or either intraepithelial carcinomas could be identified, which strongly supports the possibility of transition to malignant lesions.

Generally, the origin of epithelial ovarian carcinoma is believed to be in the ovarian surface epithelium (mesothelium) that undergoes a malignant transformation. Endometriotic cyst can also originate from the mesothelium that borders the inclusion cysts, suggesting the possibility of a common pathogenic pathway. A recent theory [24] proposes the origins of serous-type ovarian carcinoma in the epithelium of the uterine tubes, while the endometrial epithelium and stroma of the endometriotic cyst would undergo undergo malignant transformation, leading to endometrioid or a clear cell carcinomas.

The term endometriosis-related ovarian neoplasia (ERON) has been relatively recently proposed in literature as a rare but dangerous complication of endometriosis, and it includes endometrioid carcinoma, clear cell carcinoma, sero-mucinous borderline tumor, squamous cell carcinoma, Mullerian adenosarcoma, and stromal endometrioid sarcoma [25]. Recently, a study has shown that over 70% of patients with ERON had developed carcinoma in the first 10 years of monitoring, whether or not they were following a type of therapy [2]. Furthermore, atypical endometriosis lesions have been observed in 60% of cases, supporting the potential for progression and malignant transformation of these lesions [2], consistent with our findings in 19.04% of cases in Group II, probably with different amounts due to limited cases available for study selection. Moreover, the coexistence of benign, borderline, atypical, intraepithelial, and invasive neoplasia with endometriosis strongly supports the malignancy development on a background of endometriosis.

The surgical treatment may not provide a complete resection of the endometriotic lesions, especially if there are microscopic implants, nor does it prevent recurrence [26, 27]. According to several studies, endometriotic cyst excision cannot prevent the subsequent development of ovarian cancer [28, 29] and endometriotic cyst recurrence represents an additional risk factor for the development of ERON [2]. In this regard, oral contraceptives may have protective effect and, thus, may be used as a tool of primary prevention of both endometriosis and ovarian carcinoma, considering that the neoplastic transformation process diagnosis is quite difficult to be achieved in time for specific treatment.

**CONCLUSIONS**

Endometriosis is a benign condition with an incidence which is difficult to quantify due to nonspecific symptomatology and the lack of non-invasive diagnostic methods, involving young, reproductive age patients.

Diagnostic difficulties may be partially attributed to lack of specificity of the signs and symptoms, the most common being dysmenorrhea, chronic pelvic pain, and infertility. The ovarian predilection for endometriosis implantation, along with the tubal location of the implants supports the mechanism of retrograde menstruation. Moreover, the abdominal wall cutaneous post-caesarean endometriosis suggests the possibility of direct mechanical implantation, a mechanism that could also result in cervical endometriosis. An essential role is also attributed to stem cells, on a background of immunological permissive factors, in both mechanisms. Supplementary, multifocal endometriosis suggests the possibility of several mechanisms association in the etiopathogenesis of this disorder.

Endometriosis-associated lesions underline the influence of common hormonal factors and immune deficiencies in their etiopathogenesis and justify the alternative of hysterectomy as a surgical treatment addressing all these pathological spectra.

The coexistence of all spectrum of neoplasia, from benign up to invasive malignancy, with endometriosis emphasizes the need for post-operative surveillance, early identification of suspicious lesions, and development of effective prevention and therapeutic strategies.

**Conflicts of interest**

The authors declare that they have no conflict of interest.

**REFERENCES**

INTRODUCTION

Bladder tumors are frequent malignant tumors and from practical point of view they are defined as superficial and invasive. If in superficial tumors therapeutic and follow-up procedures are well standardized and the patients have a favorable prognosis, in patients with invasive urothelial carcinoma the outcome is significantly different. Although invasive tumors are more rare than superficial, the prognosis is significantly worse because they can show lymph node or/and distant metastasis in the moment of diagnosis. Chemotherapy, with or without radiotherapy, have been demonstrated as insufficient to block the tumor growth, and virtually it has no evident effect in cases with metastases.

For more than a century, researches in oncology directly and exclusively focused on malignant cells. In the last three decades there were accumulated a lot of data that support the role of tumor microenvironment not only in the local progression but also in the metastatic process. We investigated the vascular network associated to urothelial carcinoma because blood vessels are key elements in the natural evolution of malignant tumors, and the significance of microvessel density in invasive tumors shows only controversial results.

Angiogenesis is the process by which new blood vessels are formed from preexisting ones, in both normal and pathological conditions. Somehow surprising, angiogenesis was less investigated in bladder tumors, but preliminary studies have shown that the angiogenic profile of the tumor has predictive impact on local progression, lymph node and distant metastasis. There were published only few articles on MVD in urothelial carcinoma, and even few try to correlation of MVD with VEGF. In the majority of publications high MVD is associated with bad prognosis, correlating with advanced-stage, rapid local progression and metastasis. On the other hand, MVD does not bring information about the potential response to antivascular therapy, and the relation between MVD and angiogenic growth factor is uncertain.

VEGF is a growth factor, being the strongest known angiogenic substance. VEGF is secreted by a large variety of normal cells, but it is also overexpressed by tumor cells, particularly as a consequence of hypoxia generated by rapid proliferation. VEGF promotes proliferation, differentiation, survival and migration of endothelial cells in both normal and pathological conditions. VEGF becomes active after binding specific receptors expressed by endothelial cells, and from these, the most effective is VEGFR2. Although VEGFR2 is intensely expressed by urothelial carcinoma-associated blood vessels, a major antitumor effect of specific inhibitors was not yet demonstrated [1]. In many human

ABSTRACT

Objectives: To investigate the presence or absence of correlation between the immunohistochemical expression of vascular endothelial factor (VEGF) and microvessel density (MVD), and the potential prognostic value in invasive bladder carcinoma.

Methods: VEGF expression and MVD were assessed on specimens from 50 histologically confirmed invasive bladder carcinomas. VEGF expression was estimated as 0 - no positive tumor cells, 1 - less than 10% positive tumor cells, 2 - up to 50% positive tumor cells, and 3 - over 50% positive tumor cells. MVD was calculated on slides stained with CD34 antibody. The arithmetical media were statistically processed with SPSS 17.0, Student test and chi square, and p<0.5 was considered statistically significant.

Results: VEGF was positive in 13.33% of the urothelial carcinomas, and negative in adenocarcinoma and squamous cell carcinoma. In the positive cases the intensity of reaction was weak or moderate in the tumor cells. In the tumor area only the endothelium was positive for CD34, which made it possible to calculate MVD, and to detect vascular invasion. In urothelial invasive carcinoma, the average MVD calculated was 28.6. There was no statistical correlation between MVD, VEGF and tumor prognosis. High values for MVD were correlated with vascular invasion and tumor grading.

Conclusions: We found no correlation between VEGF and MVD, or tumor prognosis. MVD is an efficient tool in assessing tumor prognosis. CD34 is a useful marker in detecting vascular invasion, and immature and intermediate blood vessels that could be targets for antivascular therapy.

Key words: bladder cancer, immunohistochemistry, microvessel density, prognosis, vascular endothelial growth factor
tumors it was found a correlation between angiogenic growth factors and microvessel density, but this is not a general rule [2].

Based on the existing data, the working hypothesis of the present study is to explain the correlation or the lack of correlation between VEGF expression and MVD, results may be useful to identify a therapeutic target in the first condition, or to find out other mechanisms for bladder tumor angiogenesis in the second. This study could be useful to refine personalized therapy and to increase the efficacy of biologic therapies.

MATERIALS AND METHODS

Patients. There were investigated 50 consecutive cases with T2-T4 invasive bladder carcinoma, aged between 54 and 76 years. Diagnosis was based on clinical, imagistic, endoscopic, and pathological procedures. In all patients it was performed radical cystectomy followed by low pressure bladder reservoir. The specimens for the present study were taken from the tumor and also included neighbor apparently normal tissue.

Primary processing. Specimens were washed in buffer saline and fixed in buffer formalin for 48 to 72 hours. Paraffin embedding was done using Thermo Shandon system. From each paraffin block there were performed multiple serial sections 3 µm thick. Sections stained with hematoxylin-eosin were used for the pathologic diagnosis and evaluation of the grading of differentiation (G).

Immunohistochemistry was performed automatically, using Leica Bond-Max system (Leica Biosystems, Newcastle upon Tyne, UK). Paraffin section were submitted to antigen retrieval for 20 minutes (Bond Epitope Retrieval Solution 2, Leica Biosystems, Newcastle Ltd). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes, and then treated with the primary antibody. The working system was the Bond Polymer Refine Detection System, and the final product of reaction was visualized with 3, 3 diamino-benzidine in brown. Nuclei were stained with hematoxylin and finally sections were mounted with Baume of Canada. We used as primary antibodies anti-CD34 (clone QBEnd10, Dako Glostrup, dilution 1:25, antigen retrieval pH6), and anti VEGF-A (clone VG-1, Santa Cruz, dilution 1:25, antigen retrieval pH8).

Microscopic evaluation and image analysis. Sections were analyzed with the microscope Zeiss Axiocam 506 (Jena, Germany). MVD was calculated based on the method proposed by Weidner (1991). In brief, two independent observers have choose three microscopic fields at low power magnification with high vascular density on section stained for CD34, for each case. The arithmetical media found at x200 magnification was the value of MVD for the respective case. Both tumor and peritumor areas were taken into account and evaluated. VEGF reaction was scored as follows: 0 – negative, no tumor cells stained, 1 – less than 10% positive tumor cells, 2 –11-50%, and 3 – over 50% positive tumor cells. The outer positive control for VEGF immunohistochemical reaction was the normal kidney that shows a strong reaction particularly at tubular level.

Statistical analysis was applied to show the relationship between MVD and VEGF expression, using SPSS17.0 soft. Student test and chi square were applied, and p<0.5 has been considered as statistically significant. A survival analysis was not done, as the follow-up was less than five years in most of the cases.

RESULTS

From 50 cases, we found 44 urothelial carcinoma, 3 adenocarcinoma, and 3 squamous cell carcinoma. In urothelial carcinoma we noticed T2 in 7cases, T3 in 12, and T4 in 27. We found G1 in 2 cases, G2 in 14, and G3 in 28 cases. All cases with adenocarcinoma and squamous cell carcinoma features were T3 G2 or G3.

VEGF immunohistochemical expression has been investigated in all cases included in the study, and evaluated based on the score mentioned before. Normal and dysplastic urothelium, and papillary proliferation associated to invasive tumors were negative in all cases where they were present. The final product of reaction noticed on the outer control slides was stained in dark brown, with cytoplasmic and granular pattern. From invasive urothelial carcinoma only 5 (13.33%) have shown positive reaction. The maximum achieved score was 3 from 6 possible points. In positive cases the reaction was weak or moderate with heterogeneous distribution in the cytoplasm of tumor cells (Figure 1). On occasion, isolated tumor cells located close to the front of proliferation were intensely stained, but the density was less than 10% of tumor cells. Excepting for tumor cells, we found scattered cells in the tumor stroma with moderate positive reaction. These cells could be macrophages, based on their morphologic features. Squamous cell carcinoma (n=3) and adenocarcinoma (n+3) were negative for VEGF. We found no significant statistic correlation between VEGF overexpression, grading or pathological form of carcinoma.

CD34 reaction was positive in the endothelium of blood vessels, some fibrocytes, and interstitial cells of the smooth muscle layer. In the tumor area only the endothelium has been found positive, and this specificity allowed us to calculate MVD. All malignant cells and non-malignant cells of the stroma were largely negative. Close to the normal urothelium we found many small blood vessels, orderly arranged, with visible lumen. The density of vessels significantly increased close to dysplastic urothelium, and vessels developed branches and tip cells between tumor cells. In some cases we noticed the immature vessels and tip cells that have the tendency to
surround small groups of tumors cells, which eventually remain included in the lumen (Figure 2).

Blood vessels from tumor area were irregular, with small diameter, narrow lumen or even not visible. All these vessels had immature characters, without perivascular cells. Peritumor blood vessels were larger, with thin wall. Vascular invasion was easy detected on slides stained for CD34 in 14 cases, the rate being significantly higher than that on routine stained preparations (14 versus 8). MVD in the tumor area was significantly lower in cases of urothelial carcinoma with extensive necrosis and in well-differentiated areas of squamous cell carcinoma.

In the cases with adenocarcinoma, we did not find significant differences between vessels from tumor and peritumor areas.

In urothelial invasive carcinoma MVD values calculated based on Weidner’s method ranged from a minimum of 11.2 to 47.9 high power field, with an average of 28.6. We found no correlation between MVD, VEGF and conventional parameters of prognosis. On the other hand, high values for MVD correlated with blood vessel invasion and grading.

Figure 1. CD34 positive blood vessels. Note the vascular invasion (a). Numerous small blood vessels close to the front of proliferation (b). Immature/intermediate CD34-positive blood vessels. Note the lumen that is narrow or even not visible (c). High density of small blood vessels (d). Magnification x400.

Figure 2. Immunohistochemical expression of VEGF in invasive urothelial carcinoma. Scattered positive macrophages in the stroma (a). Focal positive tumor cells (b). Positive reaction in tumor cells at the interface with tumor stroma (c). Strong VEGF-positive tumor cells but restricted to less than 10% (d). Magnification x400.
DISCUSSION

More than 25 years ago Folkman demonstrated that tumors cannot survive, grow or metastasize without developing their own blood vessels by tumor angiogenesis [3]. It would seem only reasonable that an increased secretion of VEGF by the tumor cells would correlate with an increased intratumoral MVD, and a greater aggressiveness of the tumor. However, the results regarding the correlation between MVD and solid tumor prognosis reported by different authors were controversial, but there might have been a possible methodological error. This issue was solved by Weidner who standardized the techniques for counting the blood vessels to assess intratumoral MVD [4].

Bladder cancer is one of the most frequent malignant tumors of the urinary system, and is usually accompanied by both local invasion and metastasis in the moment of diagnosis [5].

As the angiogenesis is the promoter of tumor growth and metastasis, the antiangiogenic therapy became the most promising anticancer therapy. VEGF is the most powerful angiogenic substance and is expressed by normal cells, and overexpressed by tumor cells. Our results showed a moderate to low heterogeneous expression in only 13.33% of the invasive urothelial carcinoma cases. Only less than 10% of isolated tumor cells in the proliferation front overexpressed VEGF. Consequently, we draw the conclusion that there is no correlation between VEGF expression and tumor stage or recurrence. In similar conditions Bamias, found that VEGF was not correlated with MVD levels [6]. MVD levels ≥ 47 (assessed with CD105 antibody) were associated with longer progression-free survival after chemotherapy, hence MVD, but not VEGF, could be a useful indicator of relapse in high risk urothelial cancer cases that underwent adjuvant chemotherapy. Inoue conducted a study to assess the prognostic value of angiogenesis factor expression (MVD, VEGF, bFGF, IL8) in invasive transitional cell carcinoma of the bladder treated with neoadjuvant chemotherapy, and radical cystectomy [7]. In the pretreatment biopsy specimen VEGF expression and MVD were statistically correlated with recurrence. After chemotherapy VEGF proved to be a better predictor of recurrence than MVD and clinical stage in invasive urothelial carcinoma. The authors hypothesize that the relative overexpression of VEGF observed within the residual tumor after chemotherapy may reflect the clonal selection that allows only the tumor cells that express high levels of VEGF to survive.

Stavropoulos and colleagues found no correlation between both VEGF and MVD with any clinicopathological features, recurrence or progression in superficial primary bladder tumors), concluding that VEGF is not efficient in predicting recurrence or progression [8]. Also the authors conclude that MVD may help to predict progression in high grade patients, but not as an independent prognostic factor. Other authors while investigating the expression of VEGF and its receptors VEGFR1 and 2 in non-invasive and muscle invasive bladder cancers, found an association between these markers and disease stage and recurrence, even if it is not statistically significant [9].

VEGF promotes angiogenesis by stimulating the proliferation and differentiation of endothelial cells, via stimulating the activation of VEGFR-2. In an experimental study, Davis used DC101, a murine specific VEGFR blocking antibody to assess its antiangiogenic effect on bladder tumors growing in nude mice [10]. In the control tumors MVD was higher at tumor periphery, where was the highest concentration of VEGFR-2 also. In this zone the angiogenesis was considered more active due to the presence of numerous smaller blood vessels. After DC101 therapy, surprisingly, MVD measured by CD105 did not decrease, in fact CD105 vessels appeared to accumulate in the tumor cores after therapy, in parallel with increased VEGFR-2 expression. The authors concluded that the results are due to increased hypoxia, and CD105 positive vessels are relatively refractory to VEGFR blocking antibody. Other authors found VEGFR expression negative in the urothelium and intensely positive in stromal blood vessels in both micropapillary urothelial carcinoma and invasive urothelial carcinoma [1]. As a consequence, they concluded that there is no antitumor effect expected for VEGFR inhibitors.

Angiotensin II type 1 receptor (AT1R) expression and high MVD correlate with early intravesical recurrence in patients with non-muscle-invasive bladder cancer [11]. AT1R antagonists (candesartan) inhibit vascular endothelial growth factor (VEGF) production and dramatically decrease lung metastasis of renal cancer by inhibiting tumor angiogenesis [12]. AT1R could be a molecular target in bladder cancer therapy.

Besides the inhibitors described above, there are also other substances that could target the new vessels, such as BAI-1 brain-specific angiogenesis inhibitor-1 [13]. BAI-1 has a strong expression in normal bladder mucosa and is negatively correlated with the expression of VEGF, with MVD and tumor stage. The authors suggest that BAI-1 may be involved in the negative regulation of microvascular proliferation, at least in bladder transitional cell carcinoma.

Tumor angiogenesis and its onset mechanisms depend not only on the tumor type, but also on the tumor microenvironment [14]. TSP-1an extracellular matrix glycoprotein and a potent inhibitor of angiogenesis, was found to be downregulated in bladder tumors that change from an antiangiogenic to an angiogenic phenotype [15]. The authors found a positive correlation between TSP-1 stromal expression, MVD (p=0,031) and VEGF expression (p=0,001) in larger tumors (>3cm) organ confined.

Microvessel density (MVD) evaluated on slides stained with CD31, CD34, CD105, or for von Willebrand factor, represents a measure of tumor angiogenesis and is used as a prognostic indicator.

In our study we used CD34 for intratumoral and peritumoral blood vessels identification, and for calculating MVD. We found that in invasive bladder cancer MVD was between 11.2 to 47.9 / high power field (average 28.6). The blood vessels in the tumor area and in the proliferation zone were immature or intermediate.
In our study high values for MVD correlated with blood vessel invasion and grading. Similar results were reported by Canoglu [16]. They described a correlation between MVD and tumor grade, stage and prognosis. High MVD was associated with the risk of clinical progression in both superficial and invasive bladder carcinomas.

Bochner found that MVD was associated with disease progression in patients with organ-confined tumors, muscle invasive tumors, or tumors that spread to regional lymph nodes [17]. In the same year Jaeger described a correlation between intratumor MVD and the risk of occult metastasis in patients with invasive bladder carcinomas [18]. Other authors reported that there was no relationship between MVD and tumor grade or stage, but high MVD was associated with a worse prognosis, and concluded that MVD is an independent prognostic marker in invasive bladder cancer[19],[20].

The results were not the same in studies about urotheial carcinoma of the bladder conducted by Hawke [21]. They found that although there was a correlation between tumor MVD and survival, it was not statistically significant hence the assessment of tumor MVD in urothelial carcinoma of the bladder is of little clinical importance. The same results were reported by Dinney: MVD was not a prognostic marker for T1 transitional cell carcinoma [22]. Other authors suggest that the prognostic significance of neovascularization is better assessed by vascular area and shape related morphometric characteristics, whereas MVD becomes influential only with respect to overall survival of patients with muscle-invasive bladder tumors [23].

In another study that investigated hypoxia inducible factor 1 alpha (HIF-1), another proangiogenic factor in voided urine samples in bilharzial and non-bilharzial bladder cancer versus benign bladder tumor the authors also calculated intratumor MVD using CD34 antibody [24]. Even if there was a statistically significant difference between benign and malignant tumors in regard with HIF-1 positivity rate (p<0.001), and MVD had a higher score in the malignant tumors (70% versus 0% in the benign tumors) the authors found no significant relationship between HIF-1, and MVD on one hand, and stage and tumor grade on the other hand. Hypoxia also triggers the endothelin axis that has a direct effect on MVD in tumor area. In invasive bladder cancer ET1 expression correlates with MVD in organ confined tumors [25]. In these cases, the authors obtained a surprising result: a better prognosis for patients with upregulated bladder cancers.

**Conclusion**

Based on our results, we conclude that VEGF is not an efficient target for therapy in patients with invasive tumors of the urinary bladder, as only 13.3% overexpressed VEGF at protein level. No correlation was found with clinic-pathological parameters, and VEGF cannot be taken into account as individual prognostic marker or to predict the response to specific therapy with bevacizumab. Immunoreaction for CD34 is excellent to detect vascular invasion that significantly increases results obtained on routine stained slides. MVD is a useful tool for prognosis and we found correlation with vascular invasion and grading. Immature and intermediate blood vessels could be attractive targets for antivascular therapy, but further studies are necessary in this field.

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**References**


THYMUS MICROENVIRONMENT

The thymus, being a primary lymphoid organ, provides an extraordinary environment for the maturation of T cells. Differentiation of T cells occurs as they pass from the outer compartment (the cortex, consisting mostly of immature T cells) to the inner compartment (the medulla, consisting of mature T cells). Actually, the first step in maturation is the acquisition of a T cell receptor (TCR) as well as the coexpression of CD4 and CD8 (double positive T cell-DP). However, once the DP T cell stage has been reached, only a few T cells will leave the thymus and enter the circulation. The selection of the T cell is done mainly by the interaction between TCR and self-peptide major histocompatibility complex (MHC) ligands. If the immature DP T cells bind loosely with the MHC ligand this leads to death by neglect. However, if the immature DP T cells bind too tightly to the MHC ligand, these T cells would be able to produce apoptosis of self-cells resulting in autoimmune disease. They are, however, prevented from entering the circulation by a process known as negative selection. If the DP immature T cells bind to the MHC ligand and have an intensity of the response between that of neglect selection and negative selection, then these T cells will go further on to lineage differentiation. In the end, the mature T cell will either express CD4 or CD8 [1].

The T cell differentiation occurs by interaction between the TCR and the MHC ligand on the thymus epithelial cells (TEC) [2]. Also, as shown by Suniara et al. fibroblasts have been proven to play an important role in early T cell development. In the absence of mesenchymal cells and the formation of the network of fibroblasts inside the thymus, poor lymphoid development has been proven [3].

ORIGIN AND DEVELOPMENT OF MYOID CELLS

This class of cell, although its function still unknown, proves to be a constant feature in the thymus of different species of vertebrates, ranging from lungfish Neoceratodus forsteri [5] to amphibians[4], reptiles, birds and mammals [6,7,8]. Its origin is also disputed among scientists and a final decision has not yet been reached.

Myoid cells are said to resemble epithelial cells in their early stages of development [6]. Moreover, these cells are connected by desmosomes. Also, in 1982, Cooper and Tochinai cultured the thymus of a sub-Saharan frog, Xenopus. They noted that, if in early-stage-thymus, only epithelial cells could be seen, in later-
stage-thymus, both epithelial, lymphoid and myoid cells could be observed. These finding made some argue that thymic myoid cells originate by transdifferentiation of endodermal epithelial cells [8].

However, experiments involving chimera of chick and quail have contradicted the epithelial transdifferentiation hypothesis, stating that in fact these cells have a neuroectodermal origin. It was observed that when placing a quail neural tube on a chick embryo, quail myoid cells would develop in the chimeric thymus [7].

Other opinions argue for the origin of myoid cells in the muscle precursor cells of the covering mesodermum [9]. This view could be sustained by the fact that myoid cells of the thymus as well as muscle cells coexpress some proteins, such as acetylcholine receptor, tropomin T and desmin. Moreover, it was observed through real-time PCR that compared with thymic epithelial cells (TEC), thymic myoid cells have an overexpression of muscle-specific genes such as the 5 subunits of the acetylcholine receptor, MCK (muscle creatine kinase), muscle associated receptor tyrosin kinase (MuSK), rapsyn, utrophin, ErbB2, ErbB3 and tropomin T [10].

MYOID CELLS STRUCTURE

Myoid cells have common features with skeletal muscle fibers. These cells have also been described in other regions of the body, ranging from the testes (where they are involved in the contraction of semiferous tubes) [11] to the bone marrow [12]. In electron microscopy, myoid cells possess an irregular nucleus, with fine chromatin granules and a distinct nucleolus. Myofibrils are arranged around this central nucleus, giving this cell a characteristic banding. The cytoplasm of the myoid cells, with large amounts of myofibrils, has different patterns of organisation of myofibrils, ranging from concentric perinuclear arrangement to parallel-to-cell-surface arrangement. These feature could motivate the role of myoid cells (TMCs) in contraction or in the cell trafficking of the thymus [13].

Between these myofibrils, other cellular organelles are present: ribosomes, mitochondria, sarcoplasmic reticulum [6].

Immunohistochemically, TMCs express acetylcholine receptor (AChR), MCK (muscle creatine kinase), muscle associated tyrosin kinase receptor (MuSK), rapsyn, utrophin, ErbB2, ErbB3 and tropomin T [10].

To the moment, the physiological role of thymic myoid cells is unknown. However, multiple studies have shown different functions of the thymic myoid cells. They have been described to have a role in myogenesis during postnatal skeletal muscle regeneration. Not being involved in muscle repair and grown, thymic myoid cells prove to be a useful source for myoblast transfer [14]. Also, these cells are said to protect thymic epithelial cells (TECs) from apoptosis. In order to investigate the effect of myoid cells on TECs, TECs were both cultured alone and cocultured with myoid cells. It was observed that the TECs cocultured with myoid cells proved a strong decrease of annexin-V-FITC positive thymocytes [2].

MYOID CELLS AND MYASTHENIA GRAVIS

Myasthenia gravis (MG) is an autoimmune disease. The type of immunoglobulin commonly implicated in myasthenia gravis is either IgG1 or IgG3. The autoimmune nature of this disease has been proven by administration of IgG from patients with MG to healthy animals. In these animals, the characteristic of MG were by these means reproduced [15].

In about 85% of patients, the antibodies are targeted against acetylcholine receptors (AChR).[16] By blocking the AChR, these antibodies block the neuromuscular junction, resulting in further neuromuscular transmission. Shortly, fatigue and muscle weakness install (especially the function of eye muscles is altered) [17].

However, cases of myasthenia gravis patients without any anti-AChR antibodies have been reported. These type of MG is referred to as Anti-AChR negative myasthenia gravis. In these patients, antibodies bind to muscle specific kinase (MuSK). MuSK is a type of tyrosine kinase receptor. The result of the binding between the antibody and the MuSK receptor is the dispersion of AChR clusters. Thus, it can be inferred that MuSK receptor has a significant role in maintaining AChR clusters. Usually, MuSK positive patients have more severe forms of diseases than AChR positive MG patients [18].

The thymus has been proven to be the main site where the pathogenesis of myasthenia gravis starts. In patients with MG, thymectomy does not only alleviate
symptoms but also decreases the dose of required immunotherapy [19]. In a very recent clinical trial, 126 patients with AchR positive MG were placed in two groups randomly. The first group, would undergo a transternal thymectomy and receive prednisone treatment. The second group, on the other hand, would undergo medical management and receive prednisone treatment. After 3 years, the quantitative myasthenia gravis score (QMT score) was better for the first group than the second one [20].

Although myoid cells’ biological role is still unknown, they have proved to be very interesting to scientists as they might be implicated in the pathogenesis of myasthenia gravis.

FURTHER DIRECTIONS AND PERSPECTIVES

Going back to what we previously described in the thymus microenvironment section: when double positive DP T cells bind too tightly to the MHC ligand, they are prevented from entering circulation by negative selection. In myasthenia gravis, however, there is destruction of some self-structures, such as the AchRs. This means that somewhere along the negative selection process something goes wrong. However, the myoid cells in the thymus, as well as the thymic epithelial cells, both possess AchRs on their surfaces.

So, in the medulla of the thymus there is a cell (the myoid cell) with a structure so closely related to that of the muscle. T cells could bind too avidly to the AchR of the myoid cells, leading to further activation of B cells and production of IgGs that would not be able to perceive the difference between AchRs of the myoid and muscle cells (in fact, there is no difference between them).

In 1996, Wakkach et al, showed that while thymic epithelial cells (TEC) expressed AchRs similar to that of the muscular tissue, the levels of AchRs were insufficient for explaining the onset of MG [21] However, it might be the myoid cells who are implicated in the onset of MG. But, myoid cells, as proven, protect the thymic epithelial cells from apoptosis. Even if the AchR of the myoid cells might be implicated in MG’s onset, the fact that these cells further reduce the apoptosis of TECs (which also express AchRs) might contribute to the production of more IgG against similiar-to-muscle cells, resulting in the initiation of the disease or the worsening of the state of the patient.

On the other hand, another direction might be based on what Bo Hu et al found. Their team implemented Myf5-deficient mice and myogenin-deficient mice. Myf5, or myogenic factor 5, is a protein with a major role in regulating myogenesis. Myogenin also plays a key role in the development of striated skeletal muscle fibers. Myf5-deficient mice showed a partial deficiency of TMCs while myogenin-deficient mice showed a complete loss of TMCs. In this study, no TMC reappearance was seen, implying that TMCs cannot regenerate, in the absence of myogenin. This information suggest that the development of the TMC is controlled by myogenin. Thus, Myf5 and myogenin deficiency mice could be used in order to establish the implications of myoid cells in myasthenia gravis [22].

Based on what we know nowadays about the myoid cells, it seems clear that we need more data, more experimental models, and new approaches of their role(s) in normal and pathological conditions. It is important to remember that myoid cells are usually not reported on conventional biopsies of the thymus, as they are acidophilic elements hidden in the medulla. Even the ‘normal’ number of myoid cells in normal condition and myasthenia gravis is not very clear and requires detailed morphological studies. It is believed that understanding the molecular biology of myoid cells we will have a more precise landscape not only of myasthenia gravis, but maybe also of other autoimmune diseases.

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REFERENCES


A CRITICAL REVIEW ON THE DIAGNOSIS OF PRIMARY BLADDER NECK OBSTRUCTION

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ABSTRACT

Introduction. Lower urinary tract symptoms are extremely frequent in the male population independently from age. Among these, primary bladder neck obstruction (PBNO) accounts for a significant proportion of the cases in men younger than fifty. This disorder is difficult to be diagnosed, mostly due to other interference factors (e.g.: benign prostatic obstruction) and because of the lack of knowledge from urologists.

Objective. To review current national and international Guidelines and scientific publications on the diagnosis of PBNO and lower urinary tract symptoms (LUTS) in male patients, and to define an optimal diagnostic schedule for the everyday clinical practice.

Methods. All the available publications on PBNO and all the current national and international Guidelines on lower urinary tract symptoms were evaluated. A comparison among recommendations from different publications was done.

Results. Some variability was observed among different Guidelines and publications. The most appropriate and shared recommendations for the diagnosis of PBNO were identified. Each diagnostic procedure was analysed in detail; advantages and limitations were described.

Conclusions. PBNO is a heterogeneous disease, with a great variability in symptoms at first presentation. The initial suspect is based on clinical data, while diagnosis is only confirmed with invasive diagnostic procedures. Unfortunately, a unique diagnostic workflow is not possible due to the lack of actual knowledge on the precise aetiology of the disease. A better understanding of the cause/effect sequence may contribute to the identification of a more precise diagnostic approach.

Keywords. primary bladder neck obstruction, functional urology, voiding symptoms, storage symptoms, bladder outlet obstruction diagnosis, pelvic floor muscles hypertonicity

INTRODUCTION

Primary bladder neck obstruction (PBNO) is a voiding disorder of the male which consists of an inadequate relaxation of the bladder neck during micturition; an external urethral sphincter hypercontraction may be associated [1]. As a consequence, urinary flow is obstructed in the absence of any clear urologic disease (e.g.: benign prostatic enlargement or urethral stricture). At first presentation, patients usually report a wide variety of symptoms, both attributable to storage and voiding phase; urgency is the most prevalent symptom [2-4]. PBNO was traditionally considered an infrequent disorder. On the contrary, few reports identified this condition in up to 47-54% of male patients aged 18-45 years with chronic voiding dysfunction symptoms [5,6]. Its definitive aetiology has not been already identified, although numerous hypotheses have been proposed. A proper diagnosis is usually difficult: on one side symptoms are non-specific, on the other hand patients develop a mechanism of adaptation due to the chronic nature of the disease. Therefore, we decided to review the diagnostic workflow to the correct identification of PBNO in males.

MATERIALS AND METHODS

An extensive bibliographic research (PubMed/ Medline, Web of Science, and Cochrane databases) was performed. All the publications we were able to find on PBNO were evaluated. Moreover, current International Guidelines for non-neurogenic lower urinary tract symptoms [7-13] and urinary incontinence [14-16] were taken into consideration; in case of multiple editions, only the latest versions were analysed.

RESULTS

Being PBNO a heterogeneous disease, different diagnostic work-flow has been presented in the past. In this section we will discuss all the diagnostic procedures proposed in scientific publications specifically focused on PBNO. Moreover, Guidelines from the European Association of Urology, the American Urological Association, the International Consultation on Incontinence, the National Institute for Health and Care Excellence were included.

A variable degree of diagnostic procedures and tests were suggested for the proper characterization of male lower urinary tract symptoms. All the considered publications strongly suggest the execution of a deep
investigation of general and medical history; many factors (such as drugs, other diseases, previous surgery, lifestyle, etc.) may influence the normal urinary and faecal continence. Moreover, a careful characterization of the reported symptoms should always be done. Patients may in fact only focus on a predominant symptom, neglecting the complexity of the micturition discomfort or of other eventually associated disorders (e.g.: bowel or penile sensitivity alteration, pelvic/perineal pain or dysesthesia). An in-depth physical examination with digital rectal examination is required for the evaluation of abdominal, flank and pelvic objectivity. The more accurate is this initial assessment, the more probable is the precise identification of the pathogenic mechanism and the setting of a correct therapy.

Patient reported outcome measures (validated questionnaires) may help in assessing the degree of storage and voiding symptoms. Questionnaires are more useful when the reported symptoms are vague, when the physician suspects an unreliable or uncompliant patient, or to monitor disease course during therapy. The most used questionnaires are International Prostatic Symptoms Score to define LUTS [17] and International Consultation on Incontinence Modular Questionnaire to exclude urinary leakage [18,19].

Frequency-volume charts (bladder diaries) are written reports of data regarding bladder functioning during normal patient's life. These are semi-objective measurements of the frequency and severity of LUTS. An accurate and precise filling of these forms can provide more information to the urologist than too many investigations asked without a precise focus. They are useful in a better qualification of patient's reported symptoms, and in the identification of eventual discrepancies. Moreover, in many cases they represent a good support to the definition of the underlying pathological mechanism of storage LUTS. Voiding diaries should be filled for at least three consecutive days.

Urinalysis (physical, chemical, and microscopic evaluation of urine sediment) is a useful tool to search for leucocyte esterase or nitrite, pyuria, glycosuria, ketonuria or proteinuria. Frequently LUTS may be elicited by infections, diabetes or stones. This non-invasive inexpensive test may help in defining the subsequent diagnostic workflow, which may vary from patient to patient. In case of haematuria or history of heavy-smoking, urine cytology on three samples should be asked to exclude the presence of a possible – although infrequent in young subjects – transitional carcinoma of the bladder/upper urinary tract.

Blood tests are not routinely asked, and should only be reserved to patients with a suspicious of possible renal impairment (serum creatinine, urea) or prostatitis (total PSA, reactive C protein, procalcitonin).

Uroflowmetry with post-void residual urine is a non-invasive diagnostic investigation used to measure the flow and force of urine stream during micturition. Uroflowmetry is performed by urinating into a special funnel connected to a measurement device that calculates the voided volume and the rate of flow voided per second. At the end of micturition, post-void residual urine (amount of urine that eventually remains in the bladder after voiding) is measured by bladder ultrasound or by catheterization. Depending on the morphology of the curve, on the flow rate (average and maximum) and on post-void residual urine, this investigation may suggest the diagnosis and influence the following diagnostic workflow [20-22]. In case of doubtful or noncoherent results, it is advisable to repeat the exam at minimum twice.

Abdominal ultrasound might be performed to exclude the presence of urinary stones, hydronephrosis, transitional carcinoma of the bladder, etc.

Outpatient flexible urethroscopy is asked in order to exclude the presence of cicatrical urethral strictures or benign prostatic obstruction causing compression/occlusion to the urethra. It allows to identify bladder neck contracture and external urethral sphincter hypertonicity. The new generation scopes are usually well tolerated by patients, and no anaesthesia is required.

Urodynamics and video-urodynamics are invasive procedures previously shown to be effective in the assessment of LUTS and in the diagnosis of PBNO [5,23-26]. A urodynamic-based classification of PBNO was also proposed, which recognize three possible conditions: a high vesical pressure with low voiding flow, a normal vesical pressure with low voiding flow, and a delayed opening of the bladder neck [6]. The adoption of videourodynamic made urethrocytography an optional test.

Electromyographic tests were suggested [27], but to date there are no definitive data to support the clinical utility of these procedures.

**DISCUSSION**

Voiding symptoms are extremely frequent in the male population, starting from the age of thirty [28]. Usually, they are non-specific and may be not fully perceived by the patient. Benign prostatic obstruction and overactive bladder were traditionally accounted for as the two major causes of bladder voiding dysfunction. Therefore, other voiding disorders could be neglected. In this scenario, urologists are challenged in properly diagnosing voiding diseases which are considered rare.

Among all the possible urological dysfunction, PBNO is one of the most frequent in males; in previous publications, it was diagnosed in up to 47-54% of male patients aged 18-45 years with chronic voiding symptoms [5,6]. PBNO is defined as a delayed and/or incomplete bladder neck opening, resulting in a significant symptoms variability. According to literature, urinary frequency is
the most common symptom [2-4,29]. To date a definitive aetiology leading to PBNO has not been recognized [1], thus various etiopathogenetic hypotheses have been presented. The most reliable scientific theories presented are an incomplete dissolution of mesenchymal tissue or an excessive amount of connective tissue [30], an abnormal morphologic arrangement of the detrusor/ trigonal musculature [31], a sympathetic nervous system dysfunction determining an altered control at the bladder neck [32], structural changes at the bladder neck such as fibrous narrowing or hyperplasia [1]. None of these theories was proved. Therefore, a better understanding of the nature and aetiology of PBNO is required. Understanding the cause/effect sequence may contribute to the identification of a most suitable diagnostic schedule, which is essential for the definition of a proper therapeutic approach.

The performed literature review showed that the diagnostic workflow in case of bladder outlet obstruction remained substantially unchanged over the past decades. Discrepancies are due to the wide variability in symptoms and disorders; therefore, it was not possible to identify a unique diagnostic scheme. When evaluating non-neurogenic LUTS, recommendations and guidelines are only available for men over 40 years-old [12]. Thus, younger patients usually require an individualized and more extensive approach.

A general consensus exists on the administration of patient reported outcome measures, on the collection and the interpretation of bladder diaries, on a deep medical history collection and on an accurate physical examination. According to the current EAU Guidelines on Urinary Incontinence in Adults [16], the anamnestic report should always include details on type, timing, severity and reported symptoms of LUTS or urinary incontinence. This accurate evaluation allows the physician to get a general comprehension on the reported voiding dysfunction and on its possible nature (e.g.: voiding/filling phase, obstructive/non-obstructive, incontinence/retention, etc.). Moreover, it helps in deciding whether the patient should be referred to other specialists to investigate additional symptoms as pain or bowel dysfunction. Urinalysis and abdominal ultrasound are useful to exclude other urological conditions as possible determinants of the voiding disorder reported by the patient. Based on the results of these evaluations, a subsequent panel of diagnostic tests may be activated.

The most important urological procedures to diagnose PBNO are uroflowmetry (showing plateau and/or interrupted urinary flow, and reduced voided volume) and post-void residual urine (with no clear presence of a recurrent pattern). Invasive procedures such as urethrocytostoscopy are justified if uroflowmetry shows pathological results in patients with bothersome symptomatology [6]. Endoscopically, not only the internal urinary sphincter (bladder neck) but also the external urethral sphincter (rhabdosphincter) appears to be contracted.

Previous publications reported also the extensive use of urodynamic tests and voiding cystourethrography in men with a long history of urological complains [31]. On the contrary, a survey showed that only 11% of urologists in the United States routinely use these diagnostic procedures when assessing LUTS in the male [33]. In fact, the use of urodynamics and video-urodynamics is not widespread, due both to its invasiveness and to the limited additional information provided. Moreover, it has been demonstrated that this procedure has low sensitivity, specificity or predictive value as first level test [34]. Therefore, to date it is considered optional when suspecting PBNO.

Transrectal ultrasound is not useful in the assessment of PBNO, as it only provides information on prostatic size and shape which are not determinant in the etiopathogenetic mechanism of the disease. This diagnostic procedure, as well as serum total PSA test, should be avoided because not useful and potentially misleading.

To date there is no consensus on the use of neurophysiological testing such as concentric needle EMG, sacral reflex responses to electrical stimulation of penile nerves, and pudendal nerve latency. Second level imaging (e.g.: pelvic-perineal or lumbosacral spine magnetic resonance imaging) is only indicated in the suspicion of a disease of the peripheral nervous system or of the pelvic floor muscle system.

Not all the subjects reporting voiding dysfunction suggestive for PBNO should undergo all of the described diagnostic procedures. In our experience, judicious selection of the diagnostic workflow is mandatory to avoid procedures which add little information. Clinical presentation, subjectively perceived bother, entity and relevance of reported symptoms (pain, bowel, others) by each patient represent the optimal driver to decide whether it is better to deepen diagnostics or to follow a more clinical approach.

Finally, it was recently proposed that postural and kinematic impairments may directly correlate with PBNO; a pelvic floor muscles hypertonicity due to an adaptation mechanism in the pelvis may be responsible of the internal and external urethral sphincter hypercontraction [35,36]. Recent researches proved that full spine X-ray, magnetic resonance imaging of pelvis/perineum and gait analysis might provide useful information on pathological aspects in PBNO patients [29]. In this setting, neurophysiological testing may add extra information. If these preliminary data will be confirmed by further researches, it seems reasonable to imagine a multidisciplinary approach for disease management.
CONCLUSIONS

PBNO is a frequent condition in males, leading to altered micturition and significant reduction in quality of life. It is frequently misdiagnosed due to the non-specificity of the reported symptoms at first presentation. According to the available literature, different non-invasive and invasive procedures may be adopted in diagnosing PBNO. The most suitable diagnostic work-flow is still debatable. Patients diagnosed with PBNO are extremely heterogeneous, therefore a unique scheme may not be indicated. Invasive procedures should always be avoided in case they do not add clinical or pathological information useful in the subsequent therapeutic approach. Future researches are needed, as they might provide further insights from new perspectives, which appear to be indispensable for a better understanding of disease mechanisms.

REFERENCES


